

## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
United States Patent and Trademark  
Office  
Box PCT  
Washington, D.C.20231  
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

<b>Date of mailing (day/month/year)</b> 06 October 2000 (06.10.00)	
<b>International application No.</b> PCT/EP00/01524	<b>Applicant's or agent's file reference</b> PH 99004
<b>International filing date (day/month/year)</b> 09 February 2000 (09.02.00)	<b>Priority date (day/month/year)</b> 09 February 1999 (09.02.99)
<b>Applicant</b> WERR, Wolfgang	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

06 September 2000 (06.09.00)

☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Claudio Borton

Telephone No.: (41-22) 338.83.38



# PCT

## REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

**PCT/EP 00 / 01524**  
International Application No.

**03 FEB 2000**

(09. 02 2000)

International Filing Date

EUROPEAN PATENT OFFICE  
PCT INTERNATIONAL APPLICATION

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference  
(if desired) (12 characters maximum) PH 99004

<b>Box No. I TITLE OF INVENTION</b>	
A method for inhibiting the expression of target genes in plants	
<b>Box No. II APPLICANT</b>	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)	
RhoBio 14-20 rue Pierre Baizet BP 9163 69263 LYON CEDEX 09 FR	<input type="checkbox"/> This person is also inventor. Telephone No. 04.72.85.25.92 Facsimile No. 04.72.85.28.43 Teleprinter No.
State (that is, country) of nationality: FRANCE	State (that is, country) of residence: FRANCE
This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input checked="" type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box	
<b>Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)</b>	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)	
Professeur Wolfgang WERR. Institute für Entwicklungsbiologie Universität zu Köln 50923 Köln DE	This person is: <input type="checkbox"/> applicant only <input checked="" type="checkbox"/> applicant and inventor <input type="checkbox"/> inventor only (If this check-box is marked, do not fill in below.)
State (that is, country) of nationality: Germany DE	State (that is, country) of residence: Germany DE
This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box	
<input type="checkbox"/> Further applicants and/or (further) inventors are indicated on a continuation sheet.	
<b>Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE</b>	
The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: <input checked="" type="checkbox"/> agent <input type="checkbox"/> common representative	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)	
Aventis CropScience S.A. Département Propriété Industrielle 14 - 20 rue Pierre Baizet BP 9163 69263 Lyon Cedex 09 FR	Telephone No. 04.72.85.25.92 Facsimile No. 04.72.85.28.43 Teleprinter No.
<input type="checkbox"/> Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.	


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Sheet No 2

Box No.V DESIGNATION OF STATES	
The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked).	
<b>Regional Patent</b>	
<input checked="" type="checkbox"/> AP	<b>ARIPO Patent:</b> GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
<input checked="" type="checkbox"/> EA	<b>Eurasian Patent:</b> AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
<input checked="" type="checkbox"/> EP	<b>European Patent:</b> AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
<input checked="" type="checkbox"/> OA	<b>OAPI Patent:</b> BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line)
<b>National Patent (if other kind of protection or treatment desired, specify on dotted line)</b>	
<input checked="" type="checkbox"/> AE	United Arab Emirates
<input checked="" type="checkbox"/> AL	Albania
<input checked="" type="checkbox"/> AM	Armenia
<input checked="" type="checkbox"/> AT	Austria
<input checked="" type="checkbox"/> AU	Australia
<input checked="" type="checkbox"/> AZ	Azerbaijan
<input checked="" type="checkbox"/> BA	Bosnia and Herzegovina
<input checked="" type="checkbox"/> BB	Barbados
<input checked="" type="checkbox"/> BG	Bulgaria
<input checked="" type="checkbox"/> BR	Brazil
<input checked="" type="checkbox"/> BY	Belarus
<input checked="" type="checkbox"/> CA	Canada
<input checked="" type="checkbox"/> CH and LI	Switzerland and Liechtenstein
<input checked="" type="checkbox"/> CN	China
<input checked="" type="checkbox"/> CR	Costa Rica
<input checked="" type="checkbox"/> CU	Cuba
<input checked="" type="checkbox"/> CZ	Czech Republic
<input checked="" type="checkbox"/> DE	Germany
<input checked="" type="checkbox"/> DK	Denmark
<input checked="" type="checkbox"/> DM	Dominica
<input checked="" type="checkbox"/> EE	Estonia
<input checked="" type="checkbox"/> ES	Spain
<input checked="" type="checkbox"/> FI	Finland
<input checked="" type="checkbox"/> GB	United Kingdom
<input checked="" type="checkbox"/> GD	Grenada
<input checked="" type="checkbox"/> GE	Georgia
<input checked="" type="checkbox"/> GH	Ghana
<input checked="" type="checkbox"/> GM	Gambia
<input checked="" type="checkbox"/> HR	Croatia
<input checked="" type="checkbox"/> HU	Hungary
<input checked="" type="checkbox"/> ID	Indonesia
<input checked="" type="checkbox"/> IL	Israel
<input checked="" type="checkbox"/> IN	India
<input checked="" type="checkbox"/> IS	Iceland
<input checked="" type="checkbox"/> JP	Japan
<input checked="" type="checkbox"/> KE	Kenya
<input checked="" type="checkbox"/> KG	Kyrgyzstan
<input checked="" type="checkbox"/> KP	Democratic People's Republic of Korea
<input checked="" type="checkbox"/> KR	Republic of Korea
<input checked="" type="checkbox"/> KZ	Kazakhstan
<input checked="" type="checkbox"/> LC	Saint Lucia
<input checked="" type="checkbox"/> LK	Sri Lanka
<input checked="" type="checkbox"/> LR	Liberia
<input checked="" type="checkbox"/> LS	Lesotho
<input checked="" type="checkbox"/> LT	Lithuania
<input checked="" type="checkbox"/> LU	Luxembourg
<input checked="" type="checkbox"/> LV	Latvia
<input checked="" type="checkbox"/> MA	Morocco
<input checked="" type="checkbox"/> MD	Republic of Moldova
<input checked="" type="checkbox"/> MG	Madagascar
<input checked="" type="checkbox"/> MK	The former Yugoslav Republic of Macedonia
<input checked="" type="checkbox"/> MN	Mongolia
<input checked="" type="checkbox"/> MW	Malawi
<input checked="" type="checkbox"/> MX	Mexico
<input checked="" type="checkbox"/> NO	Norway
<input checked="" type="checkbox"/> NZ	New Zealand
<input checked="" type="checkbox"/> PL	Poland
<input checked="" type="checkbox"/> PT	Portugal
<input checked="" type="checkbox"/> RO	Romania
<input checked="" type="checkbox"/> RU	Russian Federation
<input checked="" type="checkbox"/> SD	Sudan
<input checked="" type="checkbox"/> SE	Sweden
<input checked="" type="checkbox"/> SG	Singapore
<input checked="" type="checkbox"/> SI	Slovenia
<input checked="" type="checkbox"/> SK	Slovakia
<input checked="" type="checkbox"/> SL	Sierra Leone
<input checked="" type="checkbox"/> TJ	Tajikistan
<input checked="" type="checkbox"/> TM	Turkmenistan
<input checked="" type="checkbox"/> TR	Turkey
<input checked="" type="checkbox"/> TT	Trinidad and Tobago
<input checked="" type="checkbox"/> TZ	United Republic of Tanzania
<input checked="" type="checkbox"/> UA	Ukraine
<input checked="" type="checkbox"/> UG	Uganda
<input checked="" type="checkbox"/> US	United States of America
<input checked="" type="checkbox"/> UZ	Uzbekistan
<input checked="" type="checkbox"/> VN	Viet Nam
<input checked="" type="checkbox"/> YU	Yugoslavia
<input checked="" type="checkbox"/> ZA	South Africa
<input checked="" type="checkbox"/> ZW	Zimbabwe
Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:	
<input type="checkbox"/>	
<input type="checkbox"/>	
<b>Precautionary Designation Statement:</b> In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)	



Sheet No 3

<b>Box No. VI PRIORITY CLAIM</b>		<input type="checkbox"/> Further priority claims are indicated in the Supplemental Box		
Filing date of earlier application (day month year)	Number of earlier application	Where earlier application is:		
		national application. country	regional application* regional Office	international application receiving Office
item (1) 09 FEB 1999 (09.02.1999)	99 420030.1	EP		
item (2)				
item (3)				
<input type="checkbox"/> The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office) identified above as item(s)				
<small>* Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.</small>				
<b>Box No. VII INTERNATIONAL SEARCHING AUTHORITY</b>				
<b>Choice of International Searching Authority (ISA)</b> <small>(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)</small>		<b>Request to use results of earlier search; reference to that search</b> <small>(if an earlier search has been carried out by or requested from the International Searching Authority)</small>		
ISA /		Date (day/month/year)	Number	Country (or regional Office)
<b>Box No. VIII CHECK LIST; LANGUAGE OF FILING</b>				
This international application contains the following number of sheets: request : 3 description (excluding sequence listing part) : 26 claims : 4 abstract : 1 drawings : sequence listing part of description : Total number of sheets : 34		This international application is accompanied by the item(s) marked below: 1. <input checked="" type="checkbox"/> fee calculation sheet 2. <input type="checkbox"/> separate signed power of attorney 3. <input type="checkbox"/> copy of general power of attorney; reference number, if any: 4. <input type="checkbox"/> statement explaining lack of signature 5. <input type="checkbox"/> priority document(s) identified in Box No. VI as item(s): 6. <input type="checkbox"/> translation of international application into (language): 7. <input type="checkbox"/> separate indications concerning deposited microorganism or other biological material 8. <input type="checkbox"/> nucleotide and/or amino acid sequence listing in computer readable form 9. <input type="checkbox"/> other (specify):		
Figure of the drawings which should accompany the abstract:		Language of filing of the international application: Anglais		
<b>Box No. IX SIGNATURE OF APPLICANT OR AGENT</b>				
<small>Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request)</small>				
 Franck Tétaz Département Propriété Industrielle				

For receiving Office use only	
1. Date of actual receipt of the purported international application: - 9 FEB. 2000 (09.02.2000)	2. Drawings: <input type="checkbox"/> received: <input type="checkbox"/> not received
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority (if two or more are competent): ISA /	6. <input type="checkbox"/> Transmittal of search copy delayed until search fee is paid.

For International Bureau use only
Date of receipt of the record copy by the International Bureau:



## PATENT COOPERATION TREATY

PCT

NOTIFICATION CONCERNING  
SUBMISSION OR TRANSMITTAL  
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

From the INTERNATIONAL BUREAU

To:

AVENTIS CROPS SCIENCE S.A.  
Département Propriété Industrielle  
Boite postale 9163  
F-69263 Lyon Cedex 09  
FRANCE

REÇU D.P.I.

10 11 2000

Date of mailing (day/month/year) 14 June 2000 (14.06.00)	
Applicant's or agent's file reference PH 99004	IMPORTANT NOTIFICATION
International application No. PCT/EP00/01524	International filing date (day/month/year) 09 February 2000 (09.02.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 09 February 1999 (09.02.99)
Applicant RHOBIO et al	

- The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
- This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.
- An asterisk(\*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
- The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
09 Febr 1999 (09.02.99)	99420030.1	EP	31 May 2000 (31.05.00)

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

I. Britel

Telephone No. (41-22) 338.83.38





# PATENT COOPERATION TREATY

WO 00/47754  
PCT/EP00/01524

**PCT**

From the INTERNATIONAL BUREAU

## NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:

AVENTIS CROPS SCIENCE S.A.  
Département Propriété Industrielle  
Boîte postale 9163  
F-69263 Lyon Cedex 09  
FRANCE

Date of mailing (day/month/year) 17 August 2000 (17.08.00)		
Applicant's or agent's file reference PH 99004		<b>IMPORTANT NOTICE</b>
International application No. PCT/EP00/01524	International filing date (day/month/year) 09 February 2000 (09.02.00)	Priority date (day/month/year) 09 February 1999 (09.02.99)
Applicant RHOBIO et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:  
**AU, KP, KR, US**

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

**AE, AL, AM, AP, AT, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EA, EE, EP, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, OA, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW**

The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on 17 August 2000 (17.08.00) under No. WO 00/47754

### REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

### REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

<p style="text-align: center;">The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland</p> <p>Facsimile No. (41-22) 740.14.35</p>	<p>Authorized officer</p> <p style="text-align: center;">J. Zahra</p> <p>Telephone No. (41-22) 338.83.38</p>
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Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF  
THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

Date of mailing (day/month/year) 17 August 2000 (17.08.00)	IMPORTANT NOTICE
Applicant's or agent's file reference PH 99004	International application No. PCT/EP00/01524
<p>The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.</p>	

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REQU D.P.I.

17 OCT. 2000

## PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

INFORMATION CONCERNING ELECTED  
OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

AVENTIS CROPS SCIENCE S.A.  
Département Propriété Industrielle  
Boite postale 9163  
F-69263 Lyon Cedex 09  
FRANCE

Date of mailing (day/month/year)

06 October 2000 (06.10.00)

Applicant's or agent's file reference

PH 99004

## IMPORTANT INFORMATION

International application No.

PCT/EP00/01524

International filing date (day/month/year)

09 February 2000 (09.02.00)

Priority date (day/month/year)

09 February 1999 (09.02.99)

Applicant

RHOBIO et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

AP : GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW

EP : AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE

National : AU, BG, CA, CN, CZ, DE, IL, JP, KP, KR, MN, NO, NZ, PL, RO, RU, SE, SK, US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

EA : AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

OA : BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

National : AE, AL, AM, AT, AZ, BA, BB, BR, BY, CH, CR, CU, DK, DM, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IN, IS, KE, KG, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MW, MX, PT, SD,  
SG, SI, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW

3. The applicant is reminded that he must enter the "national phase" before the expiration of 30 months from the priority date before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing, if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

The entry into the European regional phase is postponed until 31 months from the priority date for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:

Claudio Borton

Telephone No. (41-22) 338.83.38

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PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/82, A01H 5/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/47754</b> <b>(43) International Publication Date:</b> 17 August 2000 (17.08.00)
<b>(21) International Application Number:</b> PCT/EP00/01524 <b>(22) International Filing Date:</b> 9 February 2000 (09.02.00)  <b>(30) Priority Data:</b> 99420030.1      9 February 1999 (09.02.99)      EP  <b>(71) Applicant (for all designated States except US):</b> RHOBIO [FR/FR]; 14-20, rue Pierre Baizet, BP 9163, F-69263 Lyon Cedex 09 (FR).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> WERR, Wolfgang [DE/DE]; Institute für Entwicklungsbiologie, Universität zu Köln, D-50923 Köln (DE).  <b>(74) Agent:</b> AVENTIS CROPS SCIENCE S.A.; Département Propriété Industrielle, Boîte postale 9163, F-69263 Lyon Cedex 09 (FR).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <div data-bbox="1170 827 1435 1010" style="border: 1px solid black; padding: 5px; text-align: center;">RECEIVED 30 AUG 2000</div>
<b>(54) Title:</b> A METHOD FOR INHIBITING THE EXPRESSION OF TARGET GENES IN PLANTS		
<b>(57) Abstract</b> <p>This invention concerns a method for inhibiting the expression of target genes in plants wherein a chimeric DNA construct comprising at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or fragment thereof that bind to DNA or that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

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**A method for inhibiting the expression of target genes in plants**

5 The present invention relates to a method for inhibiting the expression of target genes in plants and to DNA constructs used to implement this method.

Plant transcription factors mostly belong to gene families containing numerous members. An example how large such families can grow is the *myb*-family comprised of at least 97 genes in *Arabidopsis*. Also  
10 homeobox and MADS box genes are found in multiple copies comprising several subfamilies in all plant species analyzed so far. In genetic screens for loss of function alleles only few of these genes have been associated with a mutant phenotype. One explanation for numerous silent family members may be genetic redundancy; each gene specific contributions may be subtle or  
15 dependent on special conditions. This fraction of silent genes in plant as others genomes provides a major challenge in molecular biology because these are foreseen to oppose a functional analysis also in reverse genetic approaches.

In the transformation of plants, to alter the structure and/or the function of whole plants, it may be desirable to block the expression of genes.  
20 This may be effected by altering either the sequence of the gene itself or of the transcriptional factor that controls its transcription. For that purpose the antisense technology, as described for example in the US patent 4,943,674, may be used. Site-directed mutagenesis may alternatively be used. It has for example been proposed to use in vitro altered transcriptional activators  
25 encoding proteins that bind to one or more components of the transcription complex in such a way that they competitively inhibit expression of target gene or genes in plants (EP 0 475 584). Co-suppression system may also be used to block expression of specific genes (Flavell R.B., 1994).

However these technologies require an accurate knowledge of  
30 the structure of the gene or of the transcriptional factor (namely of the DNA binding and transactivator domains) to allow the antisense sequence to specifically hybridize with the gene sequence, or to allow the mutation to only

affect some properties of the protein (DNA binding or activator domain) respectively.

Other limitations of these methods are well known by one skilled in the art : in the case of redundancy in genes and/or functions, other related  
5 genes, non altered by these targeted technologies, conceal the expression of the target gene. As a result, the observed phenotype is wild-type instead of mutant as expected. Furthermore these techniques make the isolation of new genes, or the determination of new functions difficult, sometimes impossible.

The authors of the present invention now propose an alternative  
10 approach for blocking the expression of genes in plants which meets the above requirements and presents other advantages. The method of the invention distinguishes from previous technologies in that this is, as further described, a dominant approach with high penetrance. This method is independent from any DNA sequence homology with the targeted gene, which is crucial for antisense  
15 and cosuppression approaches, and moreover circumvents the problem of redundancy in genes and/or functions.

The method of the invention involves the use of a chimeric DNA construct comprising at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or fragment  
20 thereof that bind to DNA or that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein ;

said repressor sequence being operably linked to elements allowing the transcription of said fused sequences.

The term "plant-specific sequence" means a sequence that  
25 originates from plants or has been modified (or shown) to function in plants, i.e. to actually bind to DNA in plants or to activate transcription either by binding to DNA itself or by interacting with a DNA-binding protein.

It is understood that the repressor sequence of said chimeric DNA construct preferably does not contain a sequence that codes for a second other  
30 DNA-binding domain, so that no undesirable interaction could occur.

In a preferred embodiment, said repressor sequence is at least the repressor domain of the *Drosophila engrailed* gene (*eng*).

The product of the *Drosophila engrailed (eng)* locus was shown to be involved in the regulatory interactions that govern early embryogenesis (Poole et al, 1985). Eng is a homeodomain-containing transcription factor that is required for cell fate specification through out development of the fly. A pioneering experiment was performed in *Drosophila* where the DNA-binding homeodomain of the *engrailed (eng)* gene was replaced by its *Fushi tarazu (Ftz)* counterpart, and expression of the chimeric gene controlled by heat shock promoter. After induction, the sequence specificity of the *Ftz* homeodomain directed the strong transcriptional *eng* repressor function to *Ftz* target genes resulting in phenocopies of *Ftz* loss of function alleles in transgenic progeny (John et al., 1995). Thus, the Eng protein was shown to be an active repressor in *Drosophila* (John et al, 1995) and cultured animal cells (Han & Manley, 1993). In 1996, Conlon et al use an approach in which the DNA-binding domain of a transcription activator is fused to the *engrailed* repressor domain to assist in the analysis of *Xenopus* and *Zebrafish* transcription factors (Conlon et al, 1996). But no stable transformation has been carried out until now in plants, in order to obtain transgenic plants with heritable mutant phenotype.

According to the present invention, the term "repression domain of the *Drosophila engrailed* gene (*eng*)" means a fragment of the *Drosophila engrailed* gene or of a derivative sequence thereof, said fragment comprising a nucleotide sequence that encodes a polypeptide interfering with the general transcription machinery and transcriptional activators. Said fragment advantageously comprises a minimal repression sequence coding for a polypeptide of 55 residues (Poole et al., 1985, Han K. et al., 1993). The whole *engrailed* gene may also be used but most preferably without its homeodomain.

"A derivative sequence" is understood as meaning a sequence which differs from the sequence of the *Drosophila engrailed* gene by mutation, insertion, deletion or substitution of one or more bases, or by the degeneracy of the genetic code so long as it codes for a polypeptide which is substantially the same as the Engrailed product.

Engrailed related domains, which share the same repression activity, may also be used, such as those described in Smith et al. (1996).

In another embodiment, the repressor sequence that is used may be a sequence that codes for at least the Kruppel-associated box-A (KRAB-A) domain of zinc finger proteins (Witzgall et al., 1994) for at least the RE-1-silencing transcription factor (REST) (Thiel et al., 1998), or for at least the BTB (for Broad-complex Tramtrac and Bric) domain, also known as POZ-domain (Ahmad et al., 1998 ; Huynh & Bardwell, 1998).

Chimeric DNA constructs further comprising corepressor sequences associated with said repressor sequence are also encompassed. Among the corepressors of interest, one may cite KAP-1 (Friedman et al., 1996), groucho (Tolkunova et al., 1998), or KOX-1 (Moosmann et al., 1997), or N-Cor and SMRT (Huynh and Bardwell, 1998).

In one embodiment, said repressor sequence is fused to a plant-specific sequence that encodes a DNA-binding protein domain. The term "DNA binding protein domain" refers to the whole protein that contains such a DNA-binding domain but also to parts of it independently of their ability to bind to DNA.

In a preferred embodiment, a plant transcription factor, or a DNA-binding fragment thereof, is used as the DNA-binding domain containing protein. A complete transcription factor is preferably used, as it preserves interactions between proteins involved in regulation of transcription.

In an alternative embodiment, the repressor sequence is fused to a sequence that codes for a protein or parts thereof that activates transcription by binding to a DNA-binding protein such as a transcription factor. Such proteins may either increase the affinity of DNA-binding proteins to their target sites or mediate signalling to the transcriptional initiation complex and are generally called coactivators.

In the preferred embodiment wherein the DNA-binding domain containing protein is a plant transcription factor, said transcription factor may preferably be selected from the group consisting of :

- STM ("Shootmeristemless"), member of the Knotted class of homeodomain proteins, which is an essential gene for development and function of the shoot apical meristem ;

- AP3 ("APETALA3"), member of the MADS box proteins, which is a floral organ identity B function gene essential for the development of petals and stamen. AP3 protein is known to form heterodimers with the PISTILLATA (PI) gene product.

STM and AP3 encode plant transcription factors with well-characterized loss of function alleles. Both genes are not only members of different classes of plant transcription factors but deficiencies in both genes cause phenotypic effects at different developmental stages, early in the vegetative phase or rather late in the floral development.

- ZmHox - member of the homeobox proteins, more particularly ZmHox 1a/1b and 2a/2b, which are expressed in maize meristems and proliferating cells from the early embryo to late reproductive organs. This expression pattern suggests a contribution to plant growth and morphogenesis;

- Ms-41-A and Zm-41-A. These two factors are associated with male fertility. Recent results ascertain that Ms41-A protein from *Arabidopsis* is a transcription factor belonging the family recently described as ARF1 family for Auxin Response Factor 1 (Ulmasov et al., 1997). Ms41-A analogues may also be used, such as those described in WO 97/23618 or the *Arabidopsis* gene Monopteros which encodes a transcription factor mediating embryo axis formation and vascular development (Hardtke et al., 1998), and the *Arabidopsis* gene ETTIN involved in floral development (Sessions, 1997).

The plants transformed with a chimeric DNA construct of the invention comprising such Ms-41-A factor are expected to be male sterile.

In one embodiment, the chimeric DNA construct of the invention may further comprise a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor, said sequence being in frame with the fusion construct consisting of at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or

fragment thereof that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein ;

whereby the nuclear localization of the chimeric protein, that is the product of the translation of said fusion construct is dependent on the application of said steroid hormone or analogues thereof.

The steroid hormone receptor that is used may be for example the glycocorticoid receptor (GR). In that case, dexamethasone may be used to induce the nuclear translocation of the chimeric protein.

Optionally, we can use the amino acid sequences which, when included in a protein, function to promote transport of the protein to the nucleus are known in the art and are termed nuclear localization signals (NLS).

The repression domain of the *Drosophila engrailed* gene (*eng*) is operably linked to elements allowing its expression. Such elements may more particularly comprise a promoter and polyadenylation signals.

In one embodiment, the promoter that is used is a constitutive promoter.

In another embodiment, the promoter that is used is a tissue-specific promoter or a developmentally regulated promoter. This allows a conditional loss of functions that may be desired, for example to design new plant varieties.

In still other embodiments, the promoter that is used is an inducible promoter.

The polyadenylation that can be used may be for example the 35S polyA terminator of cauliflower mosaic virus (CaMV), as disclosed in Franck et al. (1980) and NOS polyA terminator, that corresponds to the non coding 3' region of nopaline synthase of Ti plasmid of *Agrobacterium tumefaciens* (Depicker et al., 1982).

Among the preferred promoters that can be used, one can cite for example :

**a) constitutive promoters :**

- 35S promoter, or advantageously the double 35S constitutive promoter of CaMV as described in Kay et al. (1987) ;

- rice actin promoter followed by the rice actin intron (PAR-IAR) included in plasmid pAct1-F4 as described in Mc Elroy et al. (1991);
- the constitutive promoter EF-1 $\alpha$  of the gene encoding for plant elongation factor described in WO 90/02172 or in Axelos et al. (1989);
- 5       - chimeric superpromoter PSP (Ni et al., 1995) constituted by the fusion of a triple repeat of the transcriptional activity element from the promoter of the gene of *Agrobacterium tumefaciens* octopin syntase, the transcriptional activating element of the promoter of the gene *Agrobacterium tumefaciens* mannopin synthase ; and
- 10       - ubiquitin promoter from sunflower (Binet et al., 1991).

**b) specific promoters :**

• *seed-specific promoters :*

- 15       - PCRU promoter of radish cruciferin gene allowing the expression specifically in seeds, as described in Depigny-This et al. (1992) ;
- HMWG promoter (High Molecular Weight Glutenin) from barley (Anderson et al., 1989) ;
- the promoter of maize  $\gamma$ zein (P $\gamma$ zein) included in py63 plasmid in Reina et al. (1990) allowing the expression in albumen of maize
- 20       seeds ;
- PGEA1 and PGEA6 promoters corresponding to the non coding 5' region of the genes GEA1 and GEA6, expressed in the grains in *Arabidopsis thaliana* (Gaubier et al., 1993) ; and
- $\beta$ -phaseolin promoter (Riggs et al., 1989).

25       • *specific promoters which drive expression in particular plant tissues which are involved in the control of fertility :*

      Among the promoters of interest one may cite the Brassicaceae A3 or A9 promoter described in WO 92 11379, the A6 promoter described in WO 93 02197, or TA29, TA26, TA13 promoters

30       described in WO 89 10396 ;

- the Ms41-A anther-specific promoter described in WO 97/23618, which may also be used in male sterility systems ;

- a dehiscence-zone specific promoter such as the one described in EP 692 030.

5                    **c) inducible promoters :**

- a promoter inducible in stress conditions, for example heat shock, wound or interaction with pathogens (Kuhlemeier et al., 1987, WO 94/21793) ; and
- an ethanol-inducible promoter (Salter et al., 1998) ; and
- 10        - the PR1a promoter inducible by salicylic acid for example (US 5,689,044).

The DNA construct of the invention is advantageously inserted in a vector, e.g. a plasmid, for use in plant cell transformation.

15                The transformation of plant cells may be effected by transferring the above vectors in protoplasts, in particular after incubating those protoplasts in a solution of polyethyleneglycol (PEG) in the presence of divalent cations ( $\text{Ca}^{2+}$ ) as described in Krens et al. (1982).

The transformation of plant cells may also be effected by  
20        electroporation as described in Fromm et al. (1986).

A gene gun may also be used allowing the projection of metal particles coated with a DNA construct of the invention, whereby genes are delivered into cell nucleus (Sanford et al., 1988).

25                Another method for transforming plant cells is cytoplasmic or nuclear micro-injection.

In a preferred embodiment, plant cells are transformed with a DNA construct of the invention, by means of a host cell infecting said plant cells. A further subject of the present invention is thus a host cell transformed with a chimeric DNA construct as previously described. Advantageously, the  
30        above host cell is *Agrobacterium tumefaciens*, as used in particular in the methods of Bevan et al. (1984) and An (1986), or *Agrobacterium rhizogenes*, in particular as used in the method of Jouanin et al. (1987).

Plant cell transformation is preferably effected by transferring the *Agrobacterium tumefaciens* T region of an extra-chromosomal circular plasmid that induces tumors (Ti) e.g by using a binary system.

For that purpose, two vectors are constructed. In one of these  
5 vectors, the T-DNA region is removed by deletion except for the right and left borders, a marker gene being inserted between the two borders to allow the selection in plant cells. The other partner of the binary system is a helper Ti plasmid which is a modified plasmid that has no T-DNA longer but still contains the virulence genes *vir*, necessary for transforming a plant cell. This plasmid is  
10 maintained in *Agrobacterium*.

The present invention also provides a transgenic plant or parts thereof, said plant being transformed with a DNA construct of the invention, or deriving from a plant initially transformed with a DNA construct of the invention. Such transgenic plants exhibit heritable phenotypes.

15 The term "deriving" refers to plants of the following generations, as long as the parent plant is fertile.

The term "parts" of transgenic plants refer in particular to leaves, fruits, seeds, roots or cells that have been genetically transformed.

The preferred plants that are used for transformation may be for  
20 example selected from the group consisting of *Arabidopsis thaliana*, rice, tobacco, maize, Brassica, wheat, tomato and flowers (*Petunia*, rose, carnation).

The present invention thus provides a method for obtaining a transgenic plant, wherein a DNA construct of the invention is transferred and  
25 expressed in a plant cell and said cell is cultured under conditions for regenerating a whole transgenic plant.

The conditions for regenerating a whole plant from a plant cell are well-known by one skilled in the art.

30 A chimeric DNA construct of the invention may be used for inhibiting the expression of a target gene in the genome of a plant, the transcription of which involves a DNA-binding protein domain as previously defined.

Inhibiting the expression of a target gene in the genome of a plant may be desired in many purposes. Many transcription factors known by the man skilled in the art play a role in the control of metabolic pathways (starch, lipids, amino acids...) or are involved in the plant development, or in the plant sensibility to pathogen. For example, blocking a gene whose expression is necessary for pollen or another formation (e.g. the Ms-41-A transcription factor, as above described) produces male sterility. Blocking the gene controlled by the AP-3 transcription factor also leads to male sterility. As another example, blocking the gene which codes for the enzyme which catalyses the conversion of sugars to starch can be used to produced sweet corn (see EP 475 584).

It is also possible to obtain transgenic plants with enriched content in lysine by using, according to the invention, the opaque 2 transcription factor (Schmidt et al., 1990), involved in the control of the expression of certain zeins. One could further use Myb-related transcription factors involved in the control of anthocyanin biosynthesis in flowers (Martin et al., 1991 ; Matin, 1997), to modify their colour. Use of other members of Myb-related transcription factors playing a role in the regulation of phenylpropanoid and lignin biosynthesis (Tamagnone et al., 1998) could also be interesting. Some others could be involved in cellular development and senescence.

The chimeric construct, fusion of the repressor domain to the plant transcription factor or part of it, can be advantageously used to identify essential protein-protein interaction domains and interacting protein partners in planta in transgenic plants.

In a preferred embodiment, expression of a chimeric protein comprising the repressor domain in fusion to a plant transcription factor is known to cause a dominant phenocopy. By the deletion of increasing parts of plant sequences encoding the transcription factor and their repression in fusion to the repressor domain in transgenic plants, essential protein domains can be identified. These are DNA-binding domains but also others, like protein-protein interaction domains, if the transcription factor is part of multi-component complex. The method according to the invention is therefore also suitable to study protein-protein interactions in planta.

The chimeric DNA construct of the invention can be used to produce phenocopies of loss of function mutants in genes involved in transcriptional control, by reversing the biological function from activation to repression and by providing chimeric fusion proteins in excess over the endogenous function.

Phenocopy is an artificial (transgenic) situation mimicking a mutant phenotype. The term phenocopy is used by Smith et al. (1996), referring to the original description of John et al. (1995), describing a Ftz loss of function phenotype mimicked by the Eng Ftz chimeric protein.

The phenocopy caused by the expression of the chimeric fusion protein allows to associate a biological function to a given transcription factor or gene involved in transcriptional control.

A chimeric DNA construct of the invention may more particularly be used in a method for determining the function of a transcription factor in plants, comprising the steps of :

- i) fusing a sequence encoding said transcription factor to a repressor sequence to form a DNA construct as previously defined;
- ii) transforming plant cells with said DNA construct;
- iii) culturing the plants obtained from the transformed cells and observing a phenocopy of a mutation correlated with the loss of expression of genes controlled by said transcription factor.

The chimeric DNA construct of the invention allows the production of a cDNA library, said library being useful to isolate new genes controlled by the transcriptional activator as above described.

The present invention thus encompasses a method for identifying new genes in plants comprising the steps of :

- i) obtaining transgenic plants transformed with a chimeric DNA construct of the invention ;
- ii) comparing the RNA population from said transgenic plants with the RNA population of a plant that has not been transformed with a chimeric DNA construct of the invention, by amplifying (for example by Polymerase

Chain Reaction in a differential display approach (Liang et al., 1993) the RNAs repressed by expression of the chimeric DNA construct, identified as genes inactive in said transgenic plants but active in the plant that has not been transformed with a chimeric DNA construct of the invention.

5           The plants to be compared should genotypically be as identical as possible. Most preferably, the plants that have not been transformed with a chimeric DNA construct of the invention are transgenic plants transformed with a blank vector.

10           In a preferred embodiment, the method for identifying new genes in plants comprises the steps of :

          i)     obtaining transgenic plants transformed with a chimeric DNA construct comprising a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor and/or comprising an inducible promoter;

15           ii)    submitting said transgenic plants to an induction by means of a steroid hormone or analogues thereof and/or promoter inducer, whereby a phenocopy is created due to the loss of expression of target genes;

          iii)    comparing the RNA populations from said transgenic plants before and shortly after induction, by amplifying (for example by Polymerase Chain Reaction in a differential display approach (Liang et al., 1993) the RNAs repressed by expression of the chimeric DNA construct, identified as genes active before but inactive after induction.

20           In a preferred embodiment, a differential display approach associated with the AFLP technique described by Vos et al. (1995) or the PCR-Select System (Clontech) is used.

          The preferred embodiment of the method of the invention wherein an inducible system is used advantageously allows to identify direct target genes controlled by the transcriptional factor, at any time during the plant development.

30           The creation of phenocopies by means of a chimeric DNA construct of the invention presents several other advantages :

Phenocopies are hardly sensitive to genetic redundancy. In contrast to mutagenesis approaches like gene machines where knock outs of single genes only give phenotypes in non-redundant situations, the phenocopy approach is expected to be informative in redundant situations. This may be important for species which are not or not truly diploid (e.g. wheat or maize respectively). Also in many Brassica crops the genome is triplicated relative to Arabidopsis.

The advantage in redundant situations is related to the fact that the phenocopy is caused by the chimeric protein making the method independent from DNA sequence homology, crucial for antisense and cosuppression approaches, which focuses effects to single or few genes (depending on the degree of sequence similarity). The newly created chimeric repressor protein may perform and compete with all kinds of interactions and functions related to the transcription factor fused with the repressor. It thus uses functional protein domains and redundant protein functions that partly or fully compensate for the loss of a single gene product (reduced by antisense or cosuppression) therefore should be also effected by the chimeric repressor protein.

Phenocopies support results obtained in gene machines, where many additional elements in the genome frequently interfere with the conclusive association of a phenotype to a specific insertion.

The phenocopy approach is also helpful in transferring information between species, e.g. from Arabidopsis to Brassica crops or between grass crops (rice, maize, wheat, barley) and in comparing regulatory networks between species. By this way, it is possible to detect similarities and differences between groups of genes regulated by the same transcription factor within several species.

The high penetrance (80 %) and dominance of phenocopies in primary transformants distinguishes this method from lower frequency antisense and cosuppression approaches, where a few individual progeny are selected.

The below examples and figures illustrate the invention without limiting its scope in any way.

## **LEGENDS OF FIGURES**

Figure 1 represents a top view into the shoot apical meristem of a young wild-type *Arabidopsis* seedling. In addition to both cotyledons, it has developed four elaborated leaves plus two additional leaf primordia in the center covering the functional shoot apical meristem.

Figure 2 represents a top view into the shoot apical meristem of *Arabidopsis* phenocopy eng-STM of the same age as in figure 1. This phenocopy has two horizontal cotyledones and a single leaf in vertical position. A functional shoot apical meristem is missing and has presumably been consumed by initiation of the single leaf primordium.

Figure 3 represents a flower of wild-type *Arabidopsis thaliana* with outermost sepals (green), white petals, yellow stamen and central carpel.

Figure 4 represents a flower of *Arabidopsis thaliana* phenocopy eng-AP3. A single sepal has been removed frontally, two neighbouring normal sepals are slightly displaced left and right to allow insight into the second and third floral whorls. Petals and stamen are obviously replaced by sepaloid organs close to the central carpel and small filamentous structures, one visible at the bottom between the two sepaloid petals.

Figure 5 represents a eng-STM construct.

Figure 6 represents a eng-AP3 construct.

## **EXAMPLES**

### **Example 1 : Construct eng-STM**

#### **Creation of pRT $\Omega$ eng**

The cDNA clone that is used is D<sub>2</sub>B clone as described in Poole et al, 1985. This clone was obtained by inserting a 2 kb EcoRI fragment of the engrailed cDNA in a pEMBL vector. The engrailed cDNA sequence is identical to the data base entry (Genbank Access. M 10017) except for the lack of the 3'

end downstream the EcoRI site gaattc, i.e the lack of the 3' end from nucleotide 2014 included.

From the cDNA clone D<sub>2</sub>B clone a 929 Bp AflII-BamHI fragment was isolated and inserted into NotI/BamHI cleaved pRT $\Omega$ Not-Asc vector. Compatibility of the NotI/AflII sites was achieved by fill in reaction with Klenow enzyme. The cDNA fragment covers the natural translation start and 298 amino acid residues of the engrailed protein. The BamHI site or a following XbaI site can be used to create translational fusions to proteins of interest.

The construct obtained was pRT $\Omega$ eng.

The pRT $\Omega$ Not-Asc is described in Überlacker et al. (1996). It is a derivative of pRT100 (Töpfer et al., 1987) and contains the CaMV 35S promoter and a polyA signal of Cabb B.

#### ENG-STM cloning strategy

The sequence of the *STM* coding region that is used is disclosed in Long et al, 1996 and is identical to the database entry (Genbank – Access U32344).

To fuse the *STM* coding region to the engrailed repressor domain the frame of the unique XbaI cloning site in pRT  $\Omega$ eng had to be shifted which was achieved by inserting a GATCTCGA adaptor into the upstream BamHI site, which was destroyed. The *STM* coding region previously amplified by reverse transcriptase PCR from Arabidopsis RNA with an 5' terminal XbaI (upstream the translation start ATG) was inserted into the XbaI site of the adapted pRT  $\Omega$ eng. The *STM* coding sequences used contain a BamHI site immediately preceeding the natural translation stop codon, which was used subsequently to create the c-terminal GR fusion.

#### ENG-STM without homeodomain (HD)

The homeodomain of *STM* was removed in an independent experiment. For that purpose, the plasmid pRT $\Omega$  eng-STM was first partially digested with HindII, secondly to completion with BamHI and ends were ligated after fill in reaction with Klenow enzyme. Thus, the construction eng-STM ( $\Delta$ HD) was obtained.

### **Example 2 : Construct eng-AP3**

5

#### **ENG-AP3 cloning strategy**

The sequence of the AP3 coding region that is used is disclosed in Jack et al., (1992) and is identical to the data base entry (Genbank – Access – D21125).

10

The AP3 cDNA clone coding region is fused to a myc epitope at its carboxy-terminus. The protein coding region including the myc epitope was amplified by PCR with primers adding terminal BamHI sites. The resulting BamHI fragment was inserted into the BamHI site of pRTΩeng. This construct was designated as pRTΩeng-AP3.

15

### **Example 3 : Construct eng-STM-GR**

#### **ENG-STM-GR**

For fusion of the chimeric eng-STM polypeptide with the GR domain (Lloyd et al, 1994) a XhoI site in front of the TMV Ω leader was converted into a BamHI site. The eng-STM coding region was then inserted into pBI-ΔGR (Simon et al., 1996 ; Schena et al., 1991) as a BamHI fragment. The artificial BamHI site (see above) in front of the natural STM STOP codon fuses the eng-STM polypeptide in frame with the GR domain.

20

### **Example 4 : Obtention of transgenic plants**

#### **A – Arabidopsis thaliana**

Both constructs, ENG-STM and ENG-AP3, were inserted into pGPTV-Bar Asc (Überlacker et al., 1996) by use of the AscI sites flanking the expression cassette and subsequently transferred into *Agrobacterium tumefaciens*, GV3101. Infiltration of *Arabidopsis* immature inflorescences followed the protocol of Bechtold et al., (1993) with minor modifications, as follows :

25

30

A single *A. tumefaciens* colony was grown at small scale (5 ml) in liquid YEB medium (5 g beef extract, 5 g saccharose, 1 g yeast extract, 1 g bacto-tryptone, 2 mM MgSO<sub>4</sub> per liter) at 28° C for 48 hours. From this preculture 0.1 ml each are used to inoculate 4 x 500 ml LB medium (5 g yeast  
5 extract, 10 g bacto-tryptone, 10 g NaCl per liter) which are grown with vigorous shaking for 16 hours at 28°C. Bacteria are pelleted at 500 x g and resuspended in infiltration medium (50 g saccharose, 0.2 ml silvet per liter) to OD<sub>600</sub> = 0.8. Twenty plants were infiltrated for each constructs. Immature Arabidopsis inflorescences (ecotype Columbia) are therefore inserted upside down into the  
10 bacterial suspension in infiltration medium. Vacuum is produced with an oil pump for 5 minutes and released rapidly, this step is repeated 3 times. Subsequently, plants are transferred to the green house until maturation and seed harvest. Seeds are vernalized and transgenic progeny selected for BASTA (eng/stm-GR; kanamycin) resistance.

As shown on Figures 1 to 4, the phenotypes observed upon  
15 expression of eng-STM and eng-AP3 are those expected. Transgenic *eng-STM* plants exhibited abnormalities from the earliest seedling stage on. Compared to wild type (Figure 1), the petiole of this single leaf originates centrally not laterally the petioles of both cotyledons. Exceptionally shoot apical meristem  
20 (SAM) activity in *eng-STM* plants was completely terminated after initiation of a single leaf. Generally, however, subsequent primordia all develop in the same fashion and with accidental timing. These trans-dominant alterations phenocopy weak *stm* alleles and have been reproduced in various independent large-scale experiments (< 1000 transgenic T1 progeny). Generally *stm*  
25 phenocopies are found in 75 % to 90 % of the primary transformants. Alterations may be subtle early in seedling development but are manifested with increasing numbers of leave primordia until the primary SAM ultimately looses its activity in the vast majority of transgenic *eng-STM* plants.

Concomitantly or after a short break axillary meristems are  
30 activated. The resulting secondary axes exhibit weaker phenotypic alterations and often develop quite normal rosettes. A peculiar feature of these axillary meristems is switching of meristem identity back and force between vegetative and inflorescence state resulting in air-borne rosettes. The numbers of

inflorescence/vegetative cycles may vary between individual transgenic plants and exceptionally cycling may perpetuate to plant death. However, the majority of plants ultimately develop fertile flowers sometimes at terminal position. Other peculiarities are a reduced leaf size, alterations in leaf shape, fused leaves or fasciation of the inflorescence. To confirm heredity of the dominant-negative phenotype progeny of 20 T<sub>1</sub> plants exhibiting mild to strong *stm* phenocopies were analysed in the next generation. BASTA resistant T<sub>2</sub> progeny were obtained from 18 T<sub>1</sub> plants and phenocopies recovered in 16 families. The strength of phenotypic alterations and absolute numbers of affected progeny varied between different families, a variability presumably related to the expression level of the transgene, which may also account for the failure to recover phenocopies in the two residual families.

In contrast to *eng-STM* transgenic plants the phenotypic alterations caused by ectopic expression of the *eng-AP3* construct were exclusively restricted to the flower. In 70% of the primary transformants, petals were converted to sepals in the second floral whorl and filamentous structures replaced stamens in the third whorl (compare wild type in Figure 3 with transgenic plant in 4). Both homeotic transformations are reminiscent of strong *ap3* alleles and indicate that ectopic expression of the chimeric *eng-AP3* protein trans-dominantly copies the *ap3* loss of function phenotype. Due to the lack of functional stamen those *ap3*-phenocopy plants required backcrossing, but strictly segregating with the BASTA resistance marker this trans-dominant phenotype was transmitted to the next generation. The flowers from the plants transformed with the *eng-AP3* construct are male sterile.

In order to identify the chimeric *eng-AP3* protein in the cell, one could advantageously use the Myc epitope, which would be helpful to study for example whether the chimeric protein is expressed, whether it is cytoplasmatic or nuclear, whether it forms a heterodimer with the *pistilata* gene product or whether nuclear import is dependent on *pistilata*.

In summary the ectopic expression of both chimeric constructs, *eng-STM* and *eng-AP3*, results in stable, inheritable trans-dominant functions phenocopying *stm* or *ap3* loss of function alleles, respectively. Excess of

chimeric repressor transcription factor fusions can efficiently displace the native gene products from target genes.

Although expressed from a constitutive promoter both chimeric repressors eng-STM and eng-AP3 are obviously recruited by either DNA sequence specificity, protein-protein interactions or both to their natural target genes which are repressed resulting in the phenocopy of the respective loss of function mutant phenotype.

10

### **B – Maize**

15

Genetic transformation of maize, whatever the method (electroporation, Agrobacterium, microfibres, particle gun) generally involves the use of an undifferentiated cells in rapid divisions that can still regenerate into a complete plant. This type of cells constitutes the embryogenic callus (of type II) of maize. Such callus is obtained from immature embryos having the genotype HI II or A188 x B73 according to the method and the media described in Dennehey et al, 1994, and may be multiplied and maintained by successive prickings every two-weeks on the initiation medium.

20

Plants are regenerated from these callus by modifying the hormonal and osmotic equilibrium of the cells according to the method described by Vain et al, 1989.

These plants are then acclimated in greenhouse where they can be crossed or self-pollinated.

25

30

A method of genetic transformation leading to a stable integration of the modified genes in the genome of the plant is used. This method involves the use of a gene gun. The target cells are fragments of callus having a surface area of 10 to 20 mm<sup>2</sup>. Four hours before bombarding, the fragments are laid in the center of a Petri dish containing a culture medium identical to the initiation medium, further containing 0.2 M of mannitol + 0.2 M of sorbitol (16 fragments per dish). Tissues are then bombarded as previously described. The dishes are then sealed using Scellofrais® and cultivated in dark at 27°C. The first planting is effected 24 hours afterwards, then every two-weeks during three months in a medium identical to the initiation medium but containing a selective agent. The

selective agents may be for example active ingredients of herbicidal agents (Basta®, Round up®) or antibiotics (hygromycin, kanamycin).

After three months or sometimes sooner, callus that have not been inhibited by the selected agents, develop. These are usually composed of cells resulting from the division of a cell having integrated in its genome one or more copies of gene of interest. The frequency of obtaining such callus is about 0.8 callus per bombarded dish. Callus are identified, individualized, amplified and cultivated so as to regenerate plants. In order to avoid any interference with non transformed cells, all these steps are effected in culture media that contain the selective agent. The regenerated plants are acclimated and cultivated in the greenhouse where they can be crossed or self-pollinated.

In an other embodiment, the transformation of plants may be carried out with *Agrobacterium tumefaciens* and immature embryos, as described by Ishida et al., (1996).

#### **Example 5 : Expression of the endogenous STM and AP3 genes**

One explanation for these phenocopies might be homology dependent cosuppression mediated by high RNA levels of the transgene. To exclude this, transgenic eng-STM or eng-AP3 phenocopy plants were subjected to RNA gel blot and RT-PCR analysis. The chimeric transcripts derived from the CaMV 35S promoter were easily detectable in total or poly(A)<sup>+</sup> RNA from pooled seedlings or flowers (12 individual progeny) probed with the STM or AP3 coding regions. Generally the high abundance of chimeric transcripts interfered with the detection of the shorter native mRNA in these gel blot experiments. Discrimination was achieved by probing with the natural 3'UTR sequences that are lacking in the transgenes. The result obtained for the STM 3'UTR confirms transcription of the native STM gene although at a significantly reduced level.

This reduction is not due to absence of the STM or AP3 transcripts in individual T<sub>1</sub> progeny, because the native transcripts could be detected by RT-PCR experiments in each individual phenocopy plant. To

distinguish the native mRNA from the chimeric transcript a forward primers located in the protein coding region of either the STM or the AP3 transcripts was combined with reverse primers residing in both native 3' untranslated regions respectively or in the Cabb-B polyadenylation signal of pRT Not (Überlacker et al., 1996). Single tube RT-PCR experiments with three primer combinations failed to detect the endogenous transcripts because the unique forward primer achieved saturation by excess of the chimeric amplicons. In contrast the predicted PCR products, 456 bp STM and 466 bp AP3, were easily detected with the gene specific primer pairs. We have not tried to quantify these RT-PCR experiments because we wanted only to confirm presence of the native transcript in each transgenic plant, but the amount of amplicon differs between individual phenocopy plants. The level of endogenous gene transcripts is always significantly lower than in wild type, a reduction, however, which coincides with significant morphological changes in the expressing organs. In eng-AP3 transgenic flowers the appropriate organs, petals and stamen, are lacking and converted to sepals or small carpeloid filaments.

**Example 6 : Phenocopies rely on the incorporation of the chimeric eng-STM protein into the nuclear compartment**

Although the changes in size or identity of the expressing organs might explain the lower transcript levels observed in *stm* and *ap3* phenocopy plants we aimed to elaborate further evidence against homology based gene silencing. The chimeric *eng-STM* polypeptide therefore was expressed in C-terminal fusion with the hormone-binding domain of the glucocorticoid receptor in transgenic *Arabidopsis* plants. Due to this addition the resulting *eng-STM-GR* polypeptide should be cytoplasmatic and incorporated into the nuclear compartment only after hormone application. A linkage between the dexamethasone treatment and alterations in SAM activity thus should depend on the nuclear import of the chimeric *eng-STM-GR* protein and be incompatible with cosuppression.

Some of the primary transformants (T<sub>1</sub>) obtained with the *eng-STM-GR* transgene exhibited weak *stm* phenocopies. To test for

dexamethasone inducibility we focused on one T<sub>2</sub> family with phenotypically normal kanamycine resistant seedlings. After confirming their normal development to the 4-5 leaf stage 10 transgenic seedlings were sprayed with dexamethasone and photographed daily to document the developmental progress. As controls either wild-type plants treated with dexamethasone or untreated transgenic progeny were analysed over the same time interval. In the control plants, 4-6 leaf primordia newly appear within the depicted 6-day interval in these. In contrast after dexamethasone application only a single leaf primordia gets evident in the transgenic *eng-STM-GR* plants. This result was representative for 7 out of 10 transgenic progeny. The residual three transgenic progeny showed a normal development progress, however, RNA gel blot analysis revealed absence of the chimeric *eng-STM-GR* transcript, which was detected at high levels in the responsive progeny. These 3 escapes therefore are related to transgene silencing and the temporary arrest in leaf development appeared in strict correlation to the transcriptional activity of the transgene. Dexamethasone inducibility in this family, carrying a single T-DNA insertion is inherited to subsequent generations (up to T<sub>4</sub> now). But normal development is confined to heterozygous plants, while homozygous progeny often manifest a weak *stm* phenocopy late in vegetative development. We take this as an indication that the amount of the chimeric *eng-STM-GR* protein is not allowed to exceed a threshold level, an assumption also compatible with the few *stm* phenocopies observed among primary *eng-STM-GR* transformants.

Presence of the hormone in addition to the temporary arrest in SAM activity effects growth of leaf primordia. Petioles of immature leaves hardly elongated in comparison to control plant, the leaflets in *eng-STM-GR* plants consequently remain closely attached to the shoot axis. Also retarded is growth of the leaf lamina, which is often associated with leaf curling. Most of these defects in leaf development resemble observations in *stm* phenocopy plants, where initiated leaves generally grow slowly compared to wild-type plants or unaffected progeny. The only pronounced difference compared to *eng-STM* transgenic plants is the lack in petiole elongation, which, however, may be a consequence of the C-terminal fusion with the GR hormone-binding

domain. The dexamethasone inducibility strongly favours the assumption that the chimeric *eng-STM* protein exerts a dominant-negative function internally the plant cell nucleus.

5                   **Example 7 : Expression of the C-terminal deletion polypeptide *eng-STM* ( $\Delta$ HD)**

To further investigate the mechanism transgenic plants were raised expressing the C-terminal deletion polypeptide *eng-STM* ( $\Delta$ HD). This deletion protein still spans 296 aa of *STM* protein sequence including the  
10 conserved KNOX (Bürglin, 1997) and ELK domains (Kerstetter et al., 1994) but lacks the DNA-binding homeodomain. Transcription of the *eng-STM* ( $\Delta$ HD) transgene was confirmed in BASTA resistant progeny by RT-PCR resulting in the predicted 192 bp amplicon (compared to 374 bp for *eng-STM*). The obtained primary transformants exhibited *stm* phenocopies in frequency and  
15 strength indistinguishable from those observed with the full-length protein. With this construction, the trans-dominant negative function provided by the chimeric *eng-STM* protein is therefore independent of the DNA-binding activity contributed by the *STM* homeodomain.

Further results obtained in transgenic *Arabidopsis* indicate that  
20 only 50 amino acids residues of the *STM* protein are sufficient to mediate *stm* phenocopies in fusion with the *eng* N-terminus.

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### CLAIMS

1. A chimeric DNA construct comprising at least one  
5 repressor sequence in transcriptional fusion with at least one plant-specific  
sequence that codes for a protein or fragment thereof that binds to DNA or that  
activates transcription either by binding to DNA itself or by interacting with a  
DNA-binding protein ;  
said repressor sequence being operably linked to elements allowing the  
10 transcription of said fused sequences.

2. A chimeric DNA construct according to claim 1, wherein  
said repressor sequence is at least the repressor domain of the *Drosophila*  
*engrailed* gene (*eng*).  
15

3. A chimeric DNA construct according to claim 1, wherein  
said repressor sequence encodes for at least the Kruppel-associated box-A  
(KRAB-A) domain of zinc finger proteins, or for at least the RE-1-silencing  
transcription factor (REST), or for at least the BTB (for Broad-complex Tramtrac  
20 and Bric) domain, also known as POZ-domain.

4. A chimeric DNA construct according to any of claims 1 to 3,  
wherein said plant-specific sequence codes for at least a DNA-binding domain  
of a plant transcription factor.  
25

5. A chimeric DNA construct according to claim 4, wherein  
said transcription factor is selected from the group consisting of STM, AP3,  
ZmHox, Ms-41-A and Zm41-A.

6. A chimeric DNA construct according to any of claims 1 to 3,  
30 wherein said plant-specific sequence activates transcription by interacting with  
a DNA-binding protein.

7. A chimeric DNA construct according to any of claims 1 to 6, further comprising a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor, said sequence being in frame with the fusion construct consisting of at least one repressor sequence in transcriptional fusion with at least one plant-specific sequence that codes for a protein or fragment thereof that activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein ;

whereby the nuclear localization of the chimeric protein, that is the product of the translation of said fusion construct is dependent on the application of said steroid hormone or analogues thereof.

8. A chimeric DNA construct according to claim 7, wherein said hormone-binding domain of steroid hormone receptor is the hormone-binding domain of a glucocorticoid receptor.

9. A chimeric DNA construct according to any of claims 1 to 8, wherein the elements allowing the transcription of said repressor sequence comprise a constitutive promoter.

10. A host cell transformed with a DNA construct according to any of claim 1 to 9.

11. A transgenic plant or parts thereof, said plant being transformed with a DNA construct according to any of claims 1 to 9, or deriving from a plant initially transformed with a DNA construct according to any of claims 1 to 9.

12. A method for obtaining a transgenic plant according to any of claims 10 or 11, wherein a DNA construct according to any of claims 1 to 9 is transferred and expressed in a plant cell and said cell is cultured under conditions for regenerating a whole transgenic plant.

13. Use of a chimeric DNA construct according to any of claims 1 to 9 for inhibiting the expression of a target gene in the genome of a plant, the transcription of which is activated by a protein encoded by a plant-specific sequence as defined in any of claims 1 to 9.

5

14. A method for determining the function of a transcription factor in plants, comprising the steps of:

i) fusing a sequence encoding said transcription factor to a repressor sequence to form a DNA construct as defined in any of claims 4 to 9 ;

10

ii) transforming plant cells with said DNA construct;

iii) culturing the plants obtained from the transformed cells and observing a phenocopy of a mutation correlated with the loss of expression of genes controlled by said transcription factor.

15

15. A method for identifying new genes in plants, comprising the steps of :

i) obtaining transgenic plants transformed with a chimeric DNA construct according to any of claims 1 to 6 ;

20

ii) comparing the RNA population from said transgenic plants with the RNA population of a plant that has not been transformed with a chimeric DNA construct according to any of claims 1 to 6, by amplifying the RNAs repressed by expression of the chimeric DNA construct, identified as genes inactive in said transgenic plants obtained in step i) but active in the plant that has not been transformed with a chimeric DNA construct.

25

16. A method for identifying new genes in plants, comprising the steps of :

i) obtaining transgenic plants transformed with a chimeric DNA construct according to claims 7 or 8, comprising a sequence that encodes at least the hormone-binding domain of a steroid hormone receptor, and/or according to claim 9, comprising an inducible promoter ;

30

ii) submitting said transgenic plants to an induction by means of a steroid hormone or analogues thereof and/or promoter inducer, whereby a phenocopy is created due the loss of expression of target genes ;

5      iii) comparing the RNA populations from said transgenic plants before and shortly after induction, by amplifying the RNAs repressed by expression of the chimeric DNA construct, identified as genes active before but inactive after induction.

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 00/01524

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC 7 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 01313 A (BUJARD HERMANN ;GOSSEN MANFRED (US)) 18 January 1996 (1996-01-18) the whole document	1-3,6, 9-11
A	LONG J A ET AL.: "A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis" NATURE, vol. 379, 4 January 1996 (1996-01-04), pages 66-69, XP002110460 cited in the application the whole document	4,5

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the international search

30 June 2000

Date of mailing of the international search report

06/07/2000

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/01524

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RIECHMANN J L ET AL.: "DNA-binding properties of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS" NUCLEIC ACIDS RESEARCH, vol. 24, no. 16, 15 August 1996 (1996-08-15), pages 3134-3141, XP002110461 the whole document	4,5
A	WEINMANN P ET AL: "A CHIMERIC TRANSACTIVATOR ALLOWS TETRACYCLINE-RESPONSIVE GENE EXPRESSION IN WHOLE PLANTS" PLANT JOURNAL, vol. 5, no. 4, 1 January 1994 (1994-01-01), pages 559-569, XP000650341 ISSN: 0960-7412 the whole document	9-14

# INTERNATIONAL SEARCH REPORT

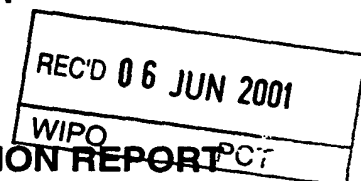
Information on patent family members

International Application No

PCT/EP 00/01524

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9601313 A	18-01-1996	US 5654168 A	05-08-1997
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		AU 3092395 A	25-01-1996
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		US 5814618 A	29-09-1998
		US 5912411 A	15-06-1999





Applicant's or agent's file reference PH 99004	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/EP00/01524	International filing date (day/month/year) 09/02/2000	Priority date (day/month/year) 09/02/1999
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant RHOBIO		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.


2. This REPORT consists of a total of 6 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☒ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand  06/09/2000	Date of completion of this report  01.06.2001
Name and mailing address of the international preliminary examining authority:   European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Bassias, I  Telephone No. +49 89 2399 8106





# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/01524

## I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):  
**Description, pages:**

1-26 as originally filed

### **Claims, No.:**

1-30 as originally filed

### **Claims, pages:**

27-30 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP00/01524

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims	2-9,13-16 (reserved opinion)
	No:	Claims	1,10-12
Inventive step (IS)	Yes:	Claims	2-6,8,9,13-16 (reserved opinion)
	No:	Claims	1,7,10-12
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	-

2. Citations and explanations  
**see separate sheet**

**VII. Certain defects in the international application**

The following defects in the form or contents of the international application have been noted:  
**see separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:  
**see separate sheet**



**R Item I**

1. The application does not contain any figures (see also Item VII, paragraph 1).

**Re Item V**

1. Reference is made to the following documents (the document numbering corresponds to their order of citation in the International Search Report):

D1: WO 96 01313 A (BUJARD HERMANN ;GOSSEN MANFRED (US)) 18 January 1996 (1996-01-18)

D4: WEINMANN P ET AL: 'A CHIMERIC TRANSACTIVATOR ALLOWS TETRACYCLINE-RESPONSIVE GENE EXPRESSION IN WHOLE PLANTS' PLANT JOURNAL, vol. 5, no. 4, 1 January 1994 (1994-01-01), pages 559-569, ISSN: 0960-7412

2. Claim 1 relates to a chimeric DNA construct comprising a repressor sequence in transcriptional fusion with a plant-specific sequence encoding a protein or a fragment thereof which binds to DNA or which activates transcription either by binding to DNA itself or by interacting with a DNA-binding protein (said repressor sequence being operably linked to elements allowing the transcription of said fused sequences).

Document D4 describes the chimeric transcriptional activator tTA which is a transcriptional fusion between the Tn10 encoded Tet repressor and the activation domain of the *Herpes simplex* virion protein VP16 under the control of the CAMV 35S promoter. Transgenic tobacco plants containing said construct were used to examine the stimulated transcription of the  $\beta$ -glucuronidase (*gus*) gene in said plants (see abstract and/or p.560, left-handed column, second paragraph). According to page 2 of the description of the present application, plant-specific sequences are defined as sequences that originate from plants or have been modified to function in plants (I.24-27).

Hence, the chimeric transcriptional activator tTA from D4 anticipates the subject-matter of claim 1 and thus said claim is not in accordance to Article 33(2) PCT.



3. Claims relating to host cells (claim 10), transgenic plants (claim 11) containing said chimeric DNA construct, or to methods for obtaining said transgenic plants (claim 12) are also not novel in view of D4 (Article 33(2) PCT).
4. Should the subject-matter of the discussed claims be novel over D4, due to minor differences, said claims would still lack an inventive activity (Article 33(3) PCT).
- 5.1. Additionally, document D1 has to be mentioned, which describes also chimeric DNA constructs comprising the tetracycline (Tet) repressor (wild-type or mutated) in transcriptional fusion with transactivator proteins which directly or indirectly (through recruitment of a transcriptional activation protein) activate transcription in eukaryotic cells (the Tet repressor is operably linked to elements [promoter] allowing the transcription of the fused DNA construct) (see p.2, l.24 - p.4, l.27 and/or section I "Tetracycline-Inducible Transcriptional Activators", p.10, l.8 - p.16, l.9). Different transactivator proteins are discussed (p.13, l.32 - p.15, l.26). However, the authors do not explicitly refer to plant-specific sequences encoding transactivator proteins. Due to this difference, it appears that in view of D1 the subject-matter of claim 1 meets the requirements of Article 33(2) PCT. However, the use of plant-specific sequences is not considered inventive since said document describes that the discussed fusion constructs can be applied in transgenic animals and **plants** (p.3, l.37-l.39 and/or p.21, l.10-l.16). Consequently, claim 1 lacks an inventive activity (Article 33(3) PCT).
- 5.2. Claims 10-12 which were lacking novelty over D4 can be objected to for lack of inventive step (Article 33(3) PCT) using D1.
- 5.3. Furthermore, D1 describes fusion constructs which contain in addition to the discussed elements a third polypeptide which promotes transport of the fusion protein to a cell nucleus (p.15, l.28 - p.16, l.9). Hence, also claim 7 does not meet the requirements of Article 33(3) PCT.
6. However, the subject-matter of the dependent claims, referring to specific repressor sequences (other than the Tet repressor) and selected plant-specific sequences are not known from the prior art. Since said specific sequences would also demonstrate the intention to use said chimeric constructs for inhibiting the



expression of target genes in plants, such claims restricted to said specific sequences could be accepted as being in accordance to Article 33(2) and (3) PCT.

All following claims being dependent or relating to such claims restricted to said specific sequences would then also meet the requirements of Article 33(2) and (3) PCT.

**Re Item VII**

1. The description of the present application contains legends of figures (p.15) and several references to said figures (e.g., p.18, l.15) although no figures have been originally filed. Consequently, the applicant is kindly requested to remove said legends and any reference to the figures from the description.
2. The reference of claim 16 to claim 9 appears to be not correct since claim 16 relates to an **inducible** promoter but claim 9 refers to a **constitutive** promoter.

**R Item VIII**

1. The use of the term "**at least**" in several claims renders the scope of the respective claims unclear (Article 6 PCT). According to the description and in particular the examples, experimental support is only given for chimeric DNA constructs comprising **one** repressor sequence in transcriptional fusion with **on** plant-specific sequence. For these reasons said term "at least" was neglected in the present examination and the applicant is requested to delete said misleading term.



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/01524

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 01313 A (BUJARD HERMANN ;GOSSEN MANFRED (US)) 18 January 1996 (1996-01-18) the whole document ---	1-3,6, 9-11
A	LONG J A ET AL.: "A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis" NATURE, vol. 379, 4 January 1996 (1996-01-04), pages 66-69, XP002110460 cited in the application the whole document --- -/--	4,5

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

30 June 2000

Date of mailing of the international search report

06/07/2000

Name and mailing address of the ISA

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Oderwald, H



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/01524

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>RIECHMANN J L ET AL.: "DNA-binding properties of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS" NUCLEIC ACIDS RESEARCH, vol. 24, no. 16, 15 August 1996 (1996-08-15), pages 3134-3141, XP002110461 the whole document</p> <p>---</p>	4,5
A	<p>WEINMANN P ET AL: "A CHIMERIC TRANSACTIVATOR ALLOWS TETRACYCLINE-RESPONSIVE GENE EXPRESSION IN WHOLE PLANTS" PLANT JOURNAL, vol. 5, no. 4, 1 January 1994 (1994-01-01), pages 559-569, XP000650341 ISSN: 0960-7412 the whole document</p> <p>-----</p>	9-14



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/01524

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9601313 A	18-01-1996	US 5654168 A	05-08-1997
		US 5789156 A	04-08-1998
		US 5866755 A	02-02-1999
		AU 3092395 A	25-01-1996
		AU 4456699 A	25-11-1999
		CA 2193122 A	18-01-1996
		CN 1167504 A	10-12-1997
		DE 804565 T	04-05-2000
		EP 0804565 A	05-11-1997
		ES 2139552 T	16-02-2000
		FI 965287 A	28-02-1997
		JP 11506901 T	22-06-1999
		NO 965623 A	28-02-1997
		US 6004941 A	21-12-1999
		US 5589362 A	31-12-1996
		US 5814618 A	29-09-1998
		US 5912411 A	15-06-1999



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# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>PH 99004</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/EP 00/ 01524</b>	International filing date (day/month/year) <b>09/02/2000</b>	(Earliest) Priority Date (day/month/year) <b>09/02/1999</b>
Applicant  <b>RHOBIO</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

**1. Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.



## INTERNATIONAL SEARCH REPORT

International Application No

EP 00/01524

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EP0-Internal, WPI Data, PAJ

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

30 June 2000

Date of mailing of the international search report

06/07/2000

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## INTERNATIONAL SEARCH REPORT

International Application No

P 00/01524

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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International Application No

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